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SAM - 109

V-32	Revised April 10, 1985	Infectious Bovine Rhinotracheitis Virus
Standard Requirement	Supersedes April 4, 1974	Agent

SUPPLEMENTAL ASSAY METHOD

FOR

TITRATION OF INFECTIOUS BOVINE RHINOTRACHEITIS NEUTRALIZING ANTIBODY BY A VIRUS PLAQUE METHOD

A. SUMMARY

This is an *in vitro* assay method which uses a cell culture system to demonstrate virus plaque reduction for determining antibody titers against Infectious Bovine Rhinotracheitis (IBR) virus.

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B. MATERIALS

1. Cell Cultures. Six well disposable plastic plates containing confluent bovine embryonic kidney (BEK) cells (2nd through 5th passage), free of extraneous agents, are used.

2. Growth Medium. The cells are grown in MEM growth medium (Appendix--1) at a temperature of 35-37 C in an incubator containing an atmosphere of 5% carbondioxide (CO_2) and a relative humidity of 70-80%.

3. Indicator Virus. APHIS-VS-BL reference IBR virus is used as the indicator virus,

4. Diluent. Maintenance medium (Appendix--2), without serum, is used to make dilutions.

C. METHODS

1. Dilution of Test Serum. The serum is heat-treated at 56 C for 30 minutes. Serial twofold dilutions are made in sterile tubes. Transfers are made with a 1 ml pipette and mixing is done with a mechanical mixer (Vortex or similar type).

   a. 0.5 ml diluent is added to tubes 2, 3, 4, and 5.

   b. 0.5 ml serum is added to tubes 1 and 2. Pipette is discarded and tube 2 is mixed. Tube 1 contains 0.5 ml of the undiluted serum, and tube 2 contains a 1:2 dilution of serum.

   c. 0.5 ml from tube 2 is transferred to tube 3. Pipette is discarded and tube 3 is mixed. Serum in tube 3 is a 1:4 dilution.

d. This process is continued until the desired number of serum dilutions are made. 0.5 ml is discarded from the last dilution tube.

2. Dilution of Indicator Virus. A vial of APHIS reference IBR virus is thawed, mixed, and diluted so that it contains 30-70 plaque forming units (PFU) per 0.1 ml.

3. Serum Neutralization of Virus and Virus Control.

a. 0.5 ml of indicator virus is added to each serum dilution tube, mixed, and held at room temperature for 45 minutes to allow for neutralization of virus. The mixing of equal volumes of serum dilution and virus results in a further two-fold dilution of serum. Thus, the undiluted serum now is a 1:2 dilution and the initial 1:2 is a 1:4, etc.

b. A virus control is prepared by mixing 1.0 ml of the indicator virus with 1.0 ml of diluent and handled in the same way as the virus-serum mixtures.

4. Inoculation of Cells and Virus Adsorption.

a. Before inoculation of the BEK cell monolayers, the growth medium is removed by aspiration with a sterile Pasteur pipette attached to a vacuum source.

b. Each well is inoculated wit 0.2 ml of a virus-serum mixture.

c. Two wells are inoculated with 0.2 ml of the virus-diluent mixture.

d. After all wells are inoculated the plate is gently rotated to spread the inoculum evenly over the surface.

e. In each series of tests, two or more wells containing cell monolayers are maintained as non-inoculated controls.

f. Inoculated cells are placed in the CO_2 incubator for one hour to allow for virus adsorption.

5. Overlay and Incubation. When the cells are ready for the overlay, 3 ml of the overlay medium (Appendix--3), at room temperature, are added to each well and the plates are returned to the CO_2 incubator. The plates remain in the incubator undisturbed for four to five days.

6. Plaque Counting. The cell cultures are prepared and counted as follows:

a. Overlay medium is removed by careful pouring.

b. One ml crystal violet solution (Appendix--4) is added to each well and allowed to spread evenly over the cell monolayer.

c. Plates are allowed to set for 10 minutes.

d. Crystal violet solution is removed by gentle washing in water and the plates dried in a 37o incubator.

e. Plaques are counted and recorded. The plaques are visible as clear circular areas in the monolayer where cells have been destroyed by the virus. Stained cell monolayers will be preserved for future reference by the buffered formalin in the crystal violet solution.

D. INTERPRETATION

The plaque reduction titer is the highest serum dilution which causes a 50% or greater reduction in the virus plaque count as compared to the average plaque count of the virus-diluent mixture.

APPENDIX

1. Growth Medium

Lactalbumin hydrolysate	0.5%	
L-Glutamine	1.0%	
MEM (Eagle with Earle's salts q.s. ad	100.00%	
Penicillin	100	units/ml
Streptomycin	100	mcg/ml
Gentomycin	50	mcg/ml
Amphotericin B (Optional)	2	mcg/ml
Fetal Calf Serum is added*	10.0%	

2. Maintenance Medium

Lactalbumin hydrolysate	0.5%	
MEM (Eagle) with Earle's salts q.s. ad	100.0%	
Penicillin	100.0	units/ml
Streptomycin	100	mcg/ml
Gentamycin	50	mcg/ml

*Fetal calf serum free of IBR virus and IBR antibodies.

3. Overlay (2 X) gum tragacanth

a. Tragacanth 2.0%

Distilled water q.s. ad 100.0%

Tragacanth is mixed with hot water by adding powder slowly with constant stirring in a blender. Fill bottles for autoclaving 1/3 full as it will boil out because of trapped air if near full. Autoclaved at 121 C for 20 minutes, cooled, and stored at 4 C.

b. Earle's Medium

Earle's BSS containing LAH and Eagle Additives*. (2X)	100.0%	
Penicillin	200	units/ml
Streptomycin	200	mcg/ml
Gentamycin	100	mcg/ml
Amphotericin B	4	mcg/ml
Fetal Calf Serum is added	4.0%	

The overlay medium is prepared just before use by combining equal parts V/V tragacanth and Earle's medium and mixing thoroughly.

4. Crystal Violet Stain 100.0%

Crystal violet powder	7.5	gn
Ethyl alcohol (70%)	5.0	ml
Formalin	25.0	ml
Dist H_2O q.s. ad	100.0	

*Eagle Additives: Vitamins, Amino Acids, and L-glutamine